TRANSCRIPTIONAL PROFILING OF POLARIZED MACROPHAGES USING RNA-SEQUENCING

A Thesis

by

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ABSTRACT

Adipose tissue macrophages (ATMs) are pivotal regulators for adipose tissue function, specifically contributing to the homeostasis of the adipose niche. Significantly increased ATMs and their altered activation patterns are causal factors to the pathogenesis of adipose tissue inflammation, and subsequently, obesity associated cardiovascular risks, type II diabetes and other metabolic syndromes. Macrophages primarily display an anti-inflammatory M2 status in lean adipose tissues whereas a proinflammatory M1 state in adipose tissues of obese individuals. Modulatory networks governing ATMs polarized activation have been investigated but the full picture remains vague. To understand the genome wide signaling networks in controlling ATM polarization, we generated transcriptome profiles from macrophages with various activation statuses- M0, M1 and M2. Among 23400 aligned unique loci from the RNA-sequencing results, around 3500 displayed differential expression pattern during macrophage polarization. The most enriched Gene Ontology terms in the category of KEGG pathways are allograft rejection and Type I diabetes mellitus pathways in M1 macrophages. IFNg was found to be one of the top upstream regulator in M1 playing pivotal role in different functional pathways. In addition, the anti-inflammatory regulator miR-223 was found to be one of top upstream regulator in M2 datasets and playing role in important functional pathways.Understanding of the complex network of interactions among different factors involved in state of polarization of macrophages would be of great advantage in finding solutions to major health issues.

DEDICATION

To my family, friends and my beloved Lord

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1. INTRODUCTION AND REVIEW OF LITERATURE *

In the last several decades, lack of balanced diet both in terms of quality and quantity has led to a rapid progression of obesity throughout the world; resulting in a major pandemic situation attracting the attention of many nations. The severity of obesity has been associated with many chronic diseases. Obese patients are prone to conditions like inflammation and insulin resistance which are causal factors in the pathogenesis of life threatening diseases like Type II Diabetes Mellitus, cardiovascular diseases and many more [1, 2, 3]. Recent studies have demonstrated that obesity is a disease status characterized as chronic, low-degree tissue inflammations, which can result from elevated infiltration of macrophages into obese tissues, and more importantly, activation status shifts from an anti-inflammatory to a proinflammatory status [1, 4, 5, 6, 7, 8, 9]. Impairment in the immune system makes it more difficult to treat these disease conditions. Macrophages are key cellular components in the innate immune system. They play an essential role in responding to invading pathogens by triggering elaborate immuno-inflammatory reactions that ultimately results in the elimination of the pathogen and reinstatement of normal conditions. In response to microenvironmental cues like pathogenic and tissue-derived molecules, macrophages undergo profound phenotypic changes and provide appropriate responses by adapting to their microenvironment [4, 10, 11]. Understanding these adaptive changes' will provide pivotal information to open the gate for development of new clinical therapies for treating chronic diseases within the proper context. Recent studies have shown that microRNAs have a profound influence on immune cell functions, including macrophage activation [12, 13].

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1.1 Macrophage Polarization and Its Diverse Functions

Macrophages play several pivotal roles in innate and adaptive immune response, tissue repair and remodeling and many more. Of the many key traits of macrophages, a major one is their functional diversity [14, 15]. This key feature could be attributed to their capability of responding to diverse stimuli and in turn exhibit diverse phenotypes and functional roles. Macrophages undergo two unique activation programs, classical (M1) and alternate (M2) activation, and a full spectrum of intermediate phenotypes between those two extremes in status [10, 14, 15]. In response to stim-

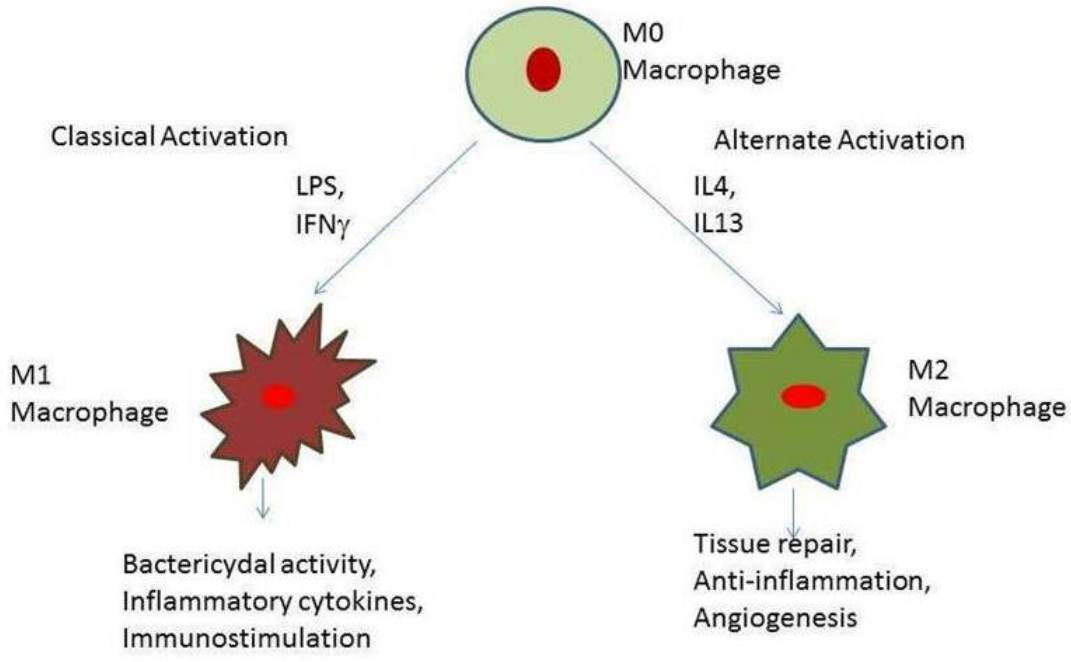

Figure 1.1: Classical and Alternative Activation In Macrophages.Schematic representation of classical versus alternative activation of macrophages depicting the stimuli, their effects on cellular function, and surrounding tissue physiology. Proinflammatory M1 macrophages contribute to bactericydal activity and production of inflammatory cytokines. Antiinflammatory M2 with induced arginase levels is a crucial component for tissue repair and angiogenesis.

uli provided by bacterial infections like lipopolysaccharide (LPS) and interferon-γ (IFN-γ), or palmitate fatty acid in the context of obesity, macrophages adopt a classical activation strategy and a proinflammatory phenotype and become highly phagocytotic, as well as exerting the bactericidal activity, and secreting proinflammatory cytokines and chemokines to further protect against invading pathogens. On the other hand, signals from interleukins(IL) like IL-4 and IL-13 promote alternative activation of macrophages which have role in parasite elimination, tissue remodeling and repair, and inhibition of tumor progression [6, 14, 16, 17] [Fig 1.1]. The phenomenon of activation of M1 and M2 macrophage polarization has been investigated intensively in recent decades as it could lead into pathways for treatment of many important disease conditions.

Genomic and transcriptional studies, and other phenotypic analyzes of M1 and M2 macrophages provided immense knowledge on several distinct characteristics extending from traits inherent in their chemokinome to metabolome [18]. For example, M1 macrophages express the Th1-attracting chemokines such as Chemokine (C-X-C Motif) Ligand 9 (CXCL9) and CXCL10, whereas M2 macrophages express the chemokines Chemokine (C-C Motif) Ligand 17 (CCL17), CCL22 and CCL24 [10, 18, 19, 20, 21, 22, 23]. In the same fashion, M1 and M2 macrophages display different functional phenotypes in response to glucose, amino acid, lipid and iron metabolism [10, 14, 15]. Even though the macrophage polarization was studied and defined in vitro using conventional methods, numerous studies have shown such polarization states in vivo, under physiological and pathological conditions. Of a note, macrophages stimulated in response to parasite infection, allergy and many tumor types resemble a large extent an M2-or M2-like phenotype [24, 25]. But it is to be considered that, in vivo conditions are often complex to interpret as other cues involved both M1 and M2 macrophages which may show a mixture of multi-

ple functional phenotypes [4, 17, 26]. Further, many study that cues which induce polarization in vivo are due to interactions among various immune and non-immune cell types like lymphocytes, dendritic cells, fibroblasts, and mesenchymal stem cells [7, 10, 17, 27] and several other factors including non-coding Ribonucleic acids (ncRNAs) [28, 29, 30]. These factors make the study on macrophage polarization in relation to these diverse cell types important. As mentioned, macrophages show functional diversity ranging from inflammation, phagocytosis, immunoregulation, tissue remodeling and even metabolism. The contribution of macrophages to inflammation is one of its most well-documented functions. In contrast to its pro-inflammatory functions, macrophages also contribute to the dampening of inflammation through their immunoregulatory properties [15, 31, 32]. Phagocytosis is a defining feature of macrophages. Macrophages not only play roles in killing pathogens but also in the elimination of dead cells and remnants of cells that is important for resolution of inflammation. In fact, it is known that phagocytosis of apoptotic cells polarizes these cells into an anti-inflammatory mode that supports their immunoregulatory functions [33].

1.2 Adipose Tissue Macrophages (ATMs) are Major Contributors to Obesity Associated Inflammation

The central feature of obesity which aggravates the progression of insulin resistance is chronic low-grade inflammation due to the infiltration of adipose tissue by macrophages [1, 4]. The dysfunction of adipose tissue with respect to maintaining energy homeostasis is associated with obesity, inflammation and metabolic complications [34]. In addition, in the case of obese people, weight loss is linked to improved insulin sensitivity and their risk of cardiovascular diseases is decreased. The inflammatory condition in obese patients is different from inflammation caused

by classical activated macrophages which are stimulated by pathogens. In response to nutrient excess and its relatively chronic in nature, adipose tissue in obese individuals has an enhanced production of pro-inflammatory cytokines, infiltration by immune related cells, especially ATMs and the formation of crown like structures where apoptotic or soon to be apoptotic adipocytes and their remnants accumulate and cluster around phagocytic macrophages [5, 35, 36]. In lean mice, the ATMs are mainly the alternatively activated M2 macrophages and when these mice were subjected to high fat diet there is seen a phenotypic switch in macrophage polarization towards a pro-inflammatory type in mouse adipose tissue [27]. Polarized macrophages play an important role in lipid metabolism and homeostasis. Studies showed ATMs from tissues of lean subjects and ATMs during weight loss to resemble M2 macrophage and found to express high levels of the anti-inflammatory cytokine IL-10 [19, 37, 38]. It is believed that these ATMs maintain adipose tissue homeostasis by protecting from inflammation in response to high-fat concentrations [39]. As explained, obesity is associated with increased accumulation of macrophages as well as with enhanced switching in polarization of ATMs from an anti-inflammatory (M2) to a pro-inflammatory (M1) state [6]. This change in polarization could be related to pathogen interference and could be specified by the targeted grouping of M1 macrophages around adipocytes that are apoptotic and having necrotic like structures [40]. Interestingly, some studies suggested that in people who are experiencing weight loss, there is a reduction in the infiltration of inflammatory macrophages into the adipose tissue and an improvement in the inflammatory response and oxidant profile of adipocytes as well as the circulating monocytes[30].

1.3 MicroRNAs are Important for Adipose Tissue Function

Compelling evidence suggested critical roles of microRNAs in regulating adipose tissue function in the context of obesity [41]. The discovery of microRNAs, a class of 21-23-nucleotide non-coding RNAs revealed a new layer of gene regulation in almost every aspect of biological processes, including those of the immune systems [12, 13]. MicroRNAs are short non-coding RNAs that are approximately 22-nucleotide in length and bind to target messenger RNAs (mRNAs) and regulate gene expression. The microRNA pairs to its target mRNAs typically result in their degradation and/or repression of translation [42]. MicroRNAs are expressed in a tissue- and cell-type specific manner and play important roles in many molecular and biological processes, including proliferation, apoptosis, development, and differentiation[12, 42, 43, 44]. During adipogenesis, microRNAs are modulating the formation and function of adipose tissue from various aspects. microRNA-33a and microRNA-33b target genes are related to metabolism [45, 46] and microRNA-103, and microRNA-107 regulate insulin sensitivity and glucose homeostasis by modulating the abundance of caveolin-1 in adipocytes [47, 48]. Furthermore, microRNAs have been associated with inflammation, oxidative stress, impaired adipogenesis and insulin signaling, and apoptosis and angiogenesis in relation to obesity. All of these processes contribute to the development of type 2 diabetes, atherosclerosis, and associated cardiovascular disorders [30, 41, 49, 50, 51]. However, their association with these processes does not necessarily imply a causal role. Each microRNA can have different roles in various conditions. For instance, microRNA-17-92 cluster, microRNA-21, microRNA-103, miR-143, microRNA-371, and miR-378/378* have shown to increase adipogenesis [47, 48, 52, 53, 54, 55, 56, 57]. This is evidenced by increased concentrations of triglycerides in circulation and enhanced expression

of adipogenic markers [33, 52, 54, 56, 57]. The microRNA-17-92 cluster induces and accelerates adipocyte differentiation by suppressing expression of the pivotal cell cycle regulator Retinobalstoma2 (Rb2/p130) [52]. In addition, let-7, microRNA-27, microRNA-130, microRNA-138, microRNA- 369-5p, and microRNA-448 inhibit adipogenesis which results in a decrease in triglycerides and down-regulation of adipogenic factors [53, 58, 59, 60]. Similarly, microRNA-21 stalls adipogenesis by inhibiting the Transforming Growth Factor beta (TGF-β) signaling pathway and microRNA-143 acts in a similar fashion through down-regulating ERK-5 function [54]. The let-7 microRNA inhibits adipogenesis by targeting high-mobility group AT-hook 2 (HMGA-2)[53], whereas microRNA-27 and microRNA-130 functions through suppressing peroxisome proliferator activated receptor γ directly [61, 62].

1.4 Significance of MicroRNAs in Regulating Adipose Tissue Macrophage Activation

MicroRNAs are now accepted as important posttranscriptional regulators of gene expression in immune cells like monocytes and macrophages [12, 13]. Varieties of inflammatory signals stimulate microRNA expression induction like LPS, Tumor Necrosis factor alpha (TNFα) or IL-1β and these tunes down TLR4/IL-1R signaling pathways in macrophages/monocytes [12, 13]. A decrease in microRNA-17, microRNA-92a and microRNA-155 is associated with an increase in monocyte/ macrophage proliferation and enhanced TLR-4 activation [63, 64, 65]. Similarly, microRNA-424 expression in endothelial cells is increased from hypoxia and switches pathway to regulate monocyte/macrophage differentiation [66, 67]. For example, different studies have shown that microRNA-146, microRNA-125b, microRNA-155 and microRNA-9 are induced by LPS and subsequently inhibiting TLR4/IL-1R signaling pathway by posttranscriptional regulation of the pathway components levels

[68, 69, 70, 71]. In a similar fashion, some studies suggested microRNAs can directly regulate production of type 2 cytokine productions during macrophage activation, for example, microRNA-98 and microRNA-21 can control the expression of IL-10 in macrophages and monocytes that in turn inhibit induction of expression of inflammatory genes [60, 72]. Recent study found that let-7c regulates bactericidal and phagocytic activities of macrophages, two functional phenotypes implicated in macrophage polarization [29]. Based on these evidences, it may be hypothesized that, in the context of obesity, the switching of inflammatory macrophages to an anti-inflammatory phenotype could be promoted by microRNAs. A study by Zhuang et.al identified that microRNA-223 acts as an important regulator of ATMs polarization and further demonstrated that it plays a significant role in modulating obesity associated insulin resistance [28]. microRNA-223 is differentially expressed during macrophage polarization, and microRNA-223deficient macrophages were hypersensitive to LPS stimulation and exhibited delayed responses to IL-4 compared with controls. Furthermore, there is an increase in M1 and decrease in M2 polarization biomarkers in microRNA-223 deficient macrophages indicated suppressive effects on activation of pro-inflammatory macrophages and a stimulatory effect on anti-inflammatory activation. microRNA-223deficient mice displayed enhanced adipose tissue inflammatory responses and decreased adipose tissue insulin signaling accompanied by inappropriate adipokine expression, which are indicators for adipose tissue dysfunction. These results support the hypothesis that microRNA-223regulated macrophage polarization, likely acting through suppressing a pro-inflammatory gene Pknox1, is important for adipose tissue function. These studies provided profound knowledge in a complex interaction in the macrophage-mediated adipose tissue inflammatory responses and metabolic regulation as well as indicating the possibility of targeting microRNAs for treatment of metabolic disorders and disease resulting from insulin resistance.

These are excerpts from the review that was published in Journal of Nutrition and Food Sciences collectively signified the importance of microRNAs in diverse roles of macrophage polarization with specific focus on the dysfunction of adipose tissue and its disorders. Impairment in the normal functioning of adipocytes leads to an inflammatory phenotype, with enhanced expression of pro-inflammatory adipocytokines and down regulation of expression of anti-inflammatory adipocytokines. The roles played by polarized macrophages are immense, and the significant contribution by microRNAs could not be ignored as well.

1.5 Transcriptional Profiling of Macrophage Polarization

ATMs are major inflammatory mediators in white adipose tissue (WAT) of obese individuals. Increased levels of chemokines secreted from adipose tissues recruit circulating macrophages and other immune cells into fat depots of obese individuals [12]. ATMs display heterogeneous activation statuses that are associated with various functions. Classic (M1) activation of ATMs leads to the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6; whereas alternative activated macrophages (M2) produce anti-inflammatory cytokines such as IL-10 and IL-13 [3]. In adipose tissue of the obese individuals, macrophages mainly display pro-inflammatory M1 responses that contribute to systemic insulin resistance [23]. Clinical studies suggest that the anti-inflammatory treatments can benefit patients with systemic insulin resistance [5]. However, major questions related to how macrophage polarized activation is regulated remain unanswered. To unveil the mechanisms underlying polarized activation of macrophages, it is necessary to generate transcriptome profiles so that valuable information can be extracted to depict the crosstalk of signaling pathways that controlling ATM responses to various stimuli. This is the major goal of my research. We adopted RNA-sequencing methodology to construct

total RNA libraries from macrophages in M0, M1, and M2 status. RNA sequencing datasets analysis revealed that several genes are differentially expressed, and signaling pathways are altered during macrophage polarization.

2. MATERIALS AND METHODS

2.1 Bone Marrow Isolation and Macrophage Differentiation

Protocols used for generation of Bone marrowderived macrophages (BMDMs) were similar to previously described [28]. In brief, bone marrow cells from wild type (WT) mice (C57Bl6) were collected. They were then treated with ammonium chloride (Stem Cell Technologies) for erythrocyte lysis, and then seeded in 100 mm plates at a concentration of $2x10^6$ cells per 1 mL. Differentiation to monocytes of cells was done with Iscoves Modified Dulbeccos Media (IMDM) medium having 10% Fetal Bovine Serum (FBS) and 15% L929 culture supernatant for 7 days. The medium was replaced on Day 3. Using flow cytometry, the generation of mature monocytes was assessed on day 7 with fluorescence-conjugated antibodies against CD11b and F4/80 [Fig 2.1a and b].

2.2 Macrophage Polarization

BMDMs were induced by lipopolysaccharide (LPS; 100 ng/mL) and Interferon gamma (IFNG; 10ng/ml) or interleukin (IL-4) (10 ng/mL) and IL13 (10ng/ml) to evaluate macrophage polarization. Using flow cytometry, surface antigens, CD69, CD80, and CD86, were observed at 24 hours after stimulation [28]. Total RNAs were extracted from these activated BMDMs at the similar time points and subjected to gene expression study.

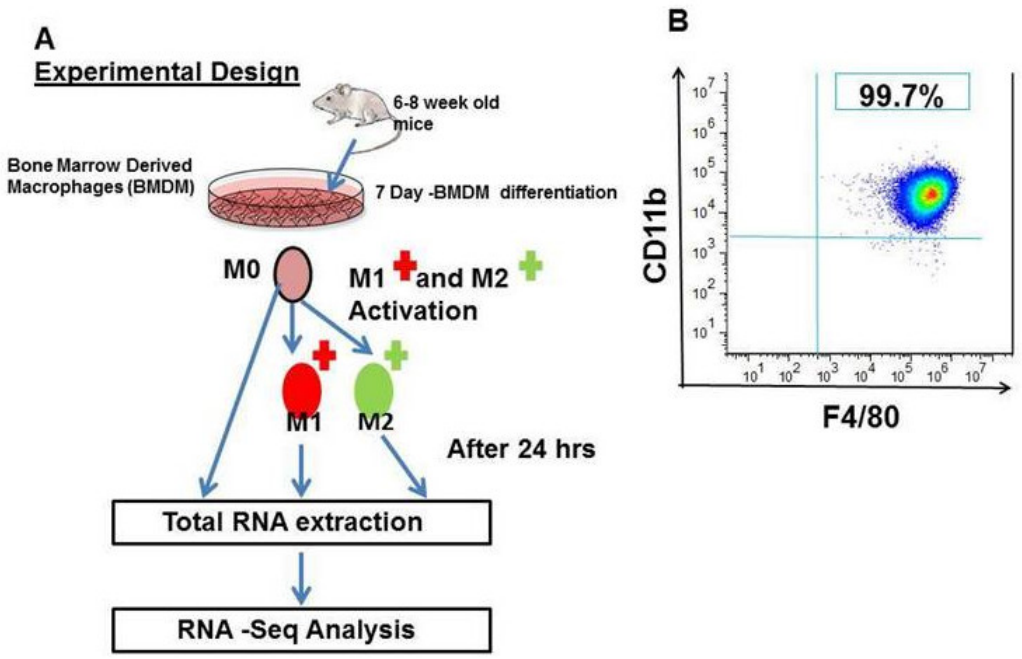

Figure 2.1: Experimental Design for In-vitro Macrophage Polarization(A). Bone marrow cells are isolated and cultured for 7 days in the presence of M-CSF. Mature macrophages are them subjected for stimulation with LPS and IFN-g for M1 and IL4 and IL13 for M2. Macrophage maturation was evaluated using flow cytometry (B). Total RNAs were extracted from activated macrophages and subjected to RNA seq analysis.

2.3 Next Generation Sequencing Data Processing

Equal amounts of RNA from LPS and IFNg treated (M1), IL4 and IL13 treated (M2) and untreated Macrophages (M0) from 5 sets of mice were pooled and the samples were used for high throughput RNA sequencing[Fig 2.2]. A total of 120 million with 40 bps single-end Illumina reads were obtained from a multiplexing run on a single lane. Reads filtered for adapter sequences and trimmed based on sequence quality (threshold of Q20) using ea-utils toolkit (https://code.google.com/p/ea-utils/). Filtered reads less than 40 bps in length was discarded. Spliced alignment was per-

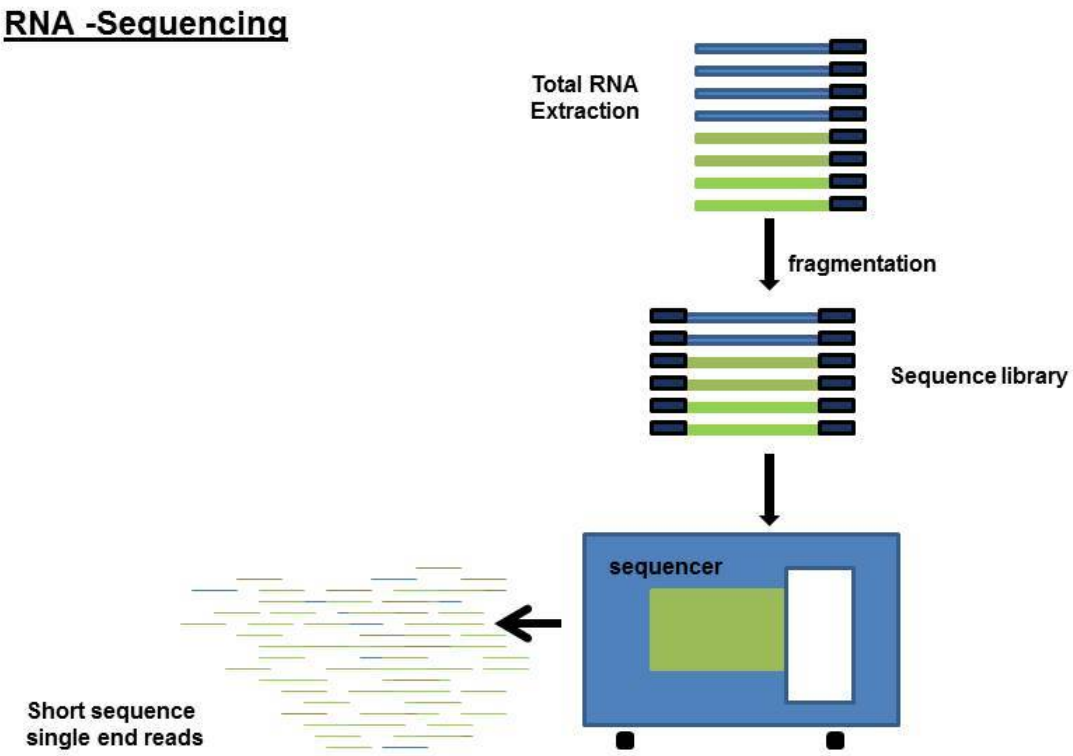

Figure 2.2: RNA-Sequencing.Briefly, long RNAs are first regenerated into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation. Sequencing adaptors (dark blue) are later on attached to each cDNA fragment and using high-throughput sequencing technology, a short sequence is generated from each cDNA.

formed against the mouse genome mus-musculus 10 (mm10) using tophat2 (TopHat 2.0.10) without allowing any mismatches to reduce the number of false positives and the resultant alignments were further processed using cufflinks2 (Cufflinks 2.2.1) to perform reference annotation based transcript assembly with bias and multi-read correction.

Differential expression analyses were performed using cuffdiff2, and the pictorial representation of it was achieved through cummeRbund (CummeRbund 2.6.1). Output data from tophat and cufflinks were converted to browser extensible data (BED)

files for viewing in the UCSC genome browser.

2.4 Gene Ontology Analyses

Functional enrichment analysis was executed using Ingenuity Pathway Analysis (IPA) version 2.0 software (Ingenuity Systems Inc., Redwood City, CA). To perform IPA analysis, all expressed genes (Fragments Per Kilobase Of Exon Model Per Million Mapped Reads (FPKM) ≥ 0.05) in M0, M1and M2 were uploaded into three columns for purpose of generating illumina probe Id and a column core analyses was performed on each dataset. As the next step, for the purpose of performing a comparison core analysis, all differentially expressed genes (fold change of 2) in M1vsM0, M2vsM0 and M1vsM2 were uploaded separately along with FPKM values and log fold change values and analyses were done. By convention, genes that were up-regulated in each dataset are shown in red and genes that were down-regulated are shown in green. During IPA analysis, by default, the molecules present in the dataset and associated with the Ingenuity Knowledge Base repository (Ingenuity Systems Inc.) were only considered. The biological functions and diseases that were most important to the dataset were identified using Functional Analysis. The significance of the association with the dataset and the specific pathways of interest were determined in three ways: (1) the activation z-score infers the activation state (Decreased or Increased), (2) a P value was calculated using Fisher's exact test which determines the probability of the association between the genes in the pathway of interest and the dataset could be explained by chance alone, and (3) ratio of the number of molecules in the dataset that mapped to the pathway to that of the total number of molecules that linked to the Ingenuity Knowledge Base pathway. Based on relationships in the molecular network, he derivations of the z-scores are calculated which represent observed causal associations in experiments between genes and those functions. Canonical pathway

study was used to identify networks that are present in the IPA library which were most significantly moderated across anatomical sites. Significance of the relationship between each dataset and the canonical pathway was evaluated in 2 ways: (1) a P value was calculated using Fisher's exact test which determines the probability that the association between the genes in the pathway of interest and the dataset could be explained by chance alone, and (2) ratio of the number of molecules in the dataset that mapped to the pathway to that of the total number of molecules that linked to the canonical pathway.

2.5 RT-qPCR

The total RNA from the isolated cells was extracted using Trizol extraction protocol according to the manufacturers instructions. For gene expression analysis, according to the manufactures protocol, quantitative RT-PCR (qPCR) was performed with iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) on Bio-Rad CFX384 (Bio-Rad). Gene specific primer pairs were used. The data obtained represents the mean of 2-$\delta\delta$Ct from at least three independent experimental repeats. Beta-actin reference gene was used for normalization [28].

2.6 Data and Statistical Analyses

Tukey post-test and One-way ANOVA were used to analyzed the dataset for each factor at individual times. Each data point represents an average of 3 technical replicates and presented as the meanSEM. Graphpad Prism version 5.01 software was used to perform the data analysis [28]. $P \leq 0.05$ was considered statistically significant.

3. RESULT AND DISCUSSION

3.1 Transcriptomic Analyses Revealed Almost Similar Number of Gene Expression

Our aim was to compare the transcriptomic expression profile of different activation states of macrophages. Accordingly, M0, the unactivated macrophages, and the two activated states Classical M1 and alternate M2 states were chosen. Total RNA was collected from these samples. The RNA sequencing of these samples was performed using the Illumina platform, generating total 40 million high-quality raw reads for each sample. The reads were aligned against the Ref-Seq mGRC38 (mouse Genome Reference Consortium 38) mm10 reference [Table 3.1] genome using TopHat. Cufflinks, a well-established transcript assembler, was used to perform the reference genome-guided transcript assembly of the aligned reads. Based on the abundance of the transcripts and their overall relative abundances that were expressed in all three types of macrophages on each chromosome were binned and were compared using cuffdiff.

Ref Seq db	mRNA	Micro RNA	lncRNA	snoRNA	snRNA	isoforms	Total Genes
Number	21754	609	2554	123	7	8801	31805

Table 3.1: MM10 Reference Database: GRCm38 (C57Bl/6J). mm10 Database was constructed by Genome Reference Consortium in 2012. It includes all 22 chromosomes. There are total 31805 transcripts which includes mRNAs, non coding RNAs and also isoforms.

The assembled transcripts and their abundances were used to perform the fol-

	Total Reads	Total Reads (After Filtering)	Total Mapped Reads	GC%	Noise
M0	33798762	32132171	95.06%	50	4.94%
M1	46233484	44123313	95.43%	50	4.57%
M2	40684911	38789883	95.21%	50	4.79%

Table 3.2: 95% of RNA Sequencing Reads Mapped. RNA-seq reads obtained are first filtered to filter out the shorter reads of less than 40bp in length, repetitive sequences and others.

lowing statistical and secondary analyses. The transcripts that were differentially expressed among the three macrophage groups and the pairwise comparison of all 3 individual samples were identified using cuffdiff. The statistical analysis is done using the square root of the Jensen-Shannon divergence computed on the relative abundances of the coding sequences, and the significance of differential expression was based on uncorrected P-value and Benjamini-Hochberg correction for multiple-testing.

Among the 40 million high quality single end raw reads for each sample, 95.06%, 95.43% and 95.21% of the reads were mapped [Table 3.2] to the mouse genome mGRC38 for M0, M1 and M2 respectively. In each of the sample, the aligned reads supported an average of 31800 transcripts including isoforms, mRNAs and non- coding RNAs (p value and FDR less than 0.05) that were identical to previously annotated mm10 database.

Interestingly, average of 23434 distinct gene loci per sample was detected, indicating the diversity introduced by isoforms of various genes. From the total of 3 sequenced samples, approximately 94000 known transcripts were identified, comprising 69012 and 26403 genes and isoforms, respectively [Fig 3.1].

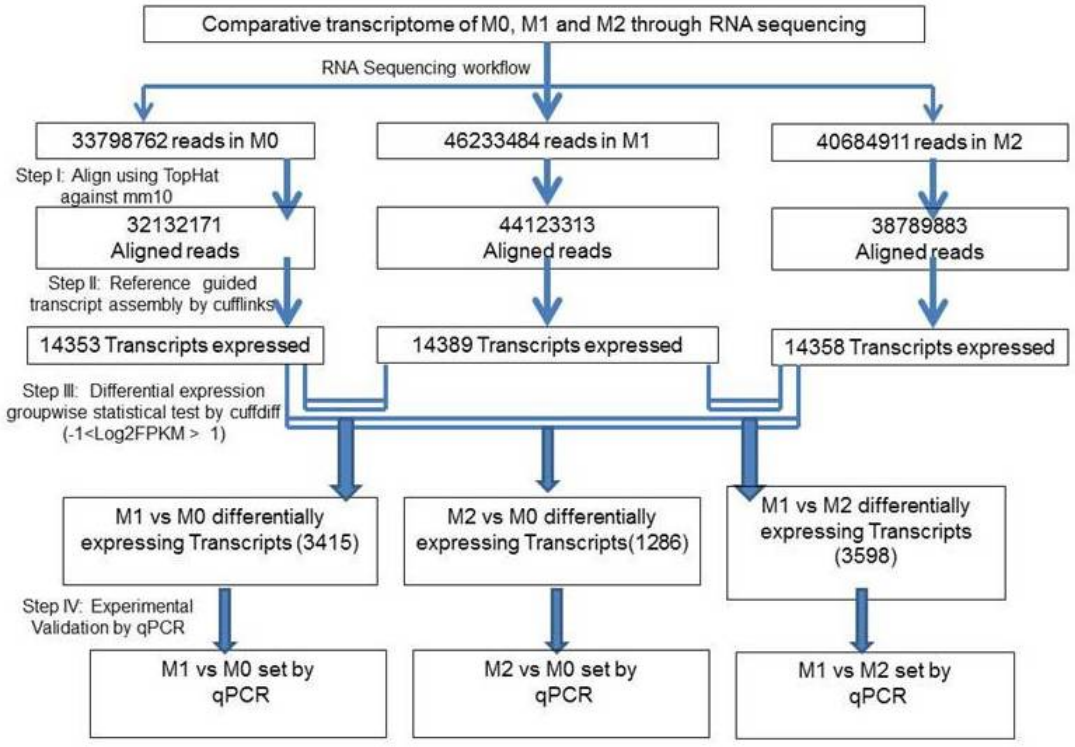

Figure 3.1: RNA-Seq Bioinformatics Analysis Workflow

With all of our sequence reads, we assessed the expression levels of genes in M0, M1 and M2 samples. Expression levels are measured in fragments per kilobase of exon model per million mapped reads (FPKM), and the sum of the FPKM values of its isoforms defines the expression level for a gene. Volcano plot showed the distribution of gene expression values is left-skewed [Fig 3.2a]; the median and mean FPKM values for M0 are 0.54836 and 34.11, respectively. Median and mean for M1 (0.5377, 45.7966) and M2(0.57498, 35.253) respectively showed the three datasets have approximately similar median.

In order to avoid all the transcripts with FPKM values that are very close to 0, we set the lower bound of 0.05 as an FPKM value in our subsequent analyses. Using this criterion, we detected 14353, 14389 and 14358 transcripts expressed in our M0,

M1 and M2 cells [Table 3.3].

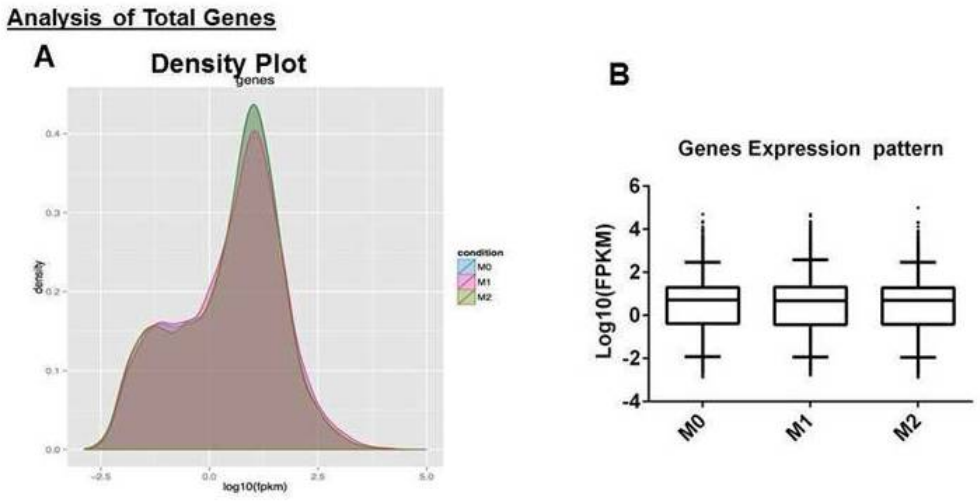

Figure 3.2: Intensity and Total Number of Expressed Genes are Comparable in Macrophages at Various Activation Status. The gene expression among M0, M1 and M2. Density plot shows that most of the genes are expressed between 0.5 and 2 value of log10(FPKM). More number of genes are expressed in M2 in the range of 0.75 to 1.5 log10(FPKM). Box plot showing distribution of the expression profiles of the three differently activated macrophages. Each box is based on 24000 genes based on their FPKM values. Median FPKM values are denoted by black solid lines and the top and bottom box edges denote the first and third quartile of FPKM values comprising genes. Whiskers represents the largest and smallest data within 1.5 times that of the interquartile range of FPKM.

Dataset	M0	M1	M2
No. of Genes Expressed (FPKM>0.05)	14353	14389	14358

Table 3.3: Genes are Considered Expressed with FPKM Value Greater than 0.05. In the three datasets, approximately 14000 genes expressed.

The transcript abundance was calculated based on the FPKM, and all expressed transcripts were further binned on the basis of their abundance (FPKM). Based on these grouping, it was revealed that the majority of the assembled transcripts were low in abundance, i.e., below 10 FPKM. We classified genes into groups based on their FPKM values: Not expressed (FPKM < 0.05), low expressed ($0.05 \leq$ FPKM < 10), medium expression ($10 \leq$ FPKM < 100), and high expression (FPKM ≥ 100) [Fig 3.3].

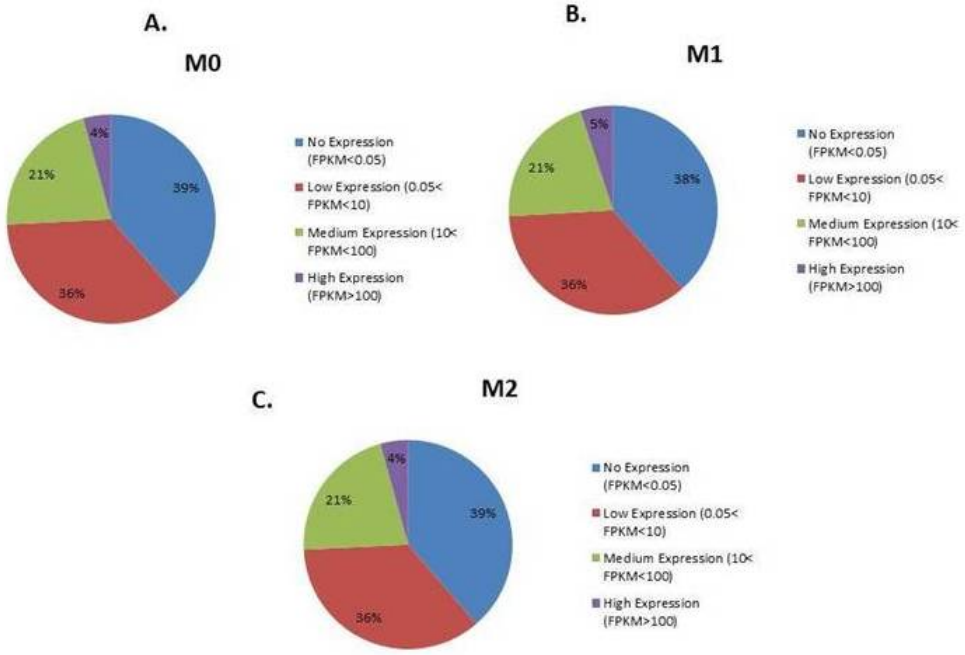

Figure 3.3: Distribution of Expression Pattern of Genes Based on FPKM. Classified genes into groups based on their FPKM values- Not expressed (FPKM < 0.05), low expressed ($0.05 \leq$ FPKM < 10), medium expression ($10 \leq$ FPKM < 100), and high expression (FPKM ≥ 100).

In general, the transcripts expressed were higher in M2 than in the other two groups. However, the overall number of transcripts and their expression profile in all

three macrophages followed a similar trend [Fig 3.2b]. In agreement with the Box plots, MA plots also showed similar outliers and similar expression pattern in all the three samples [Fig 3.4a,b and c].

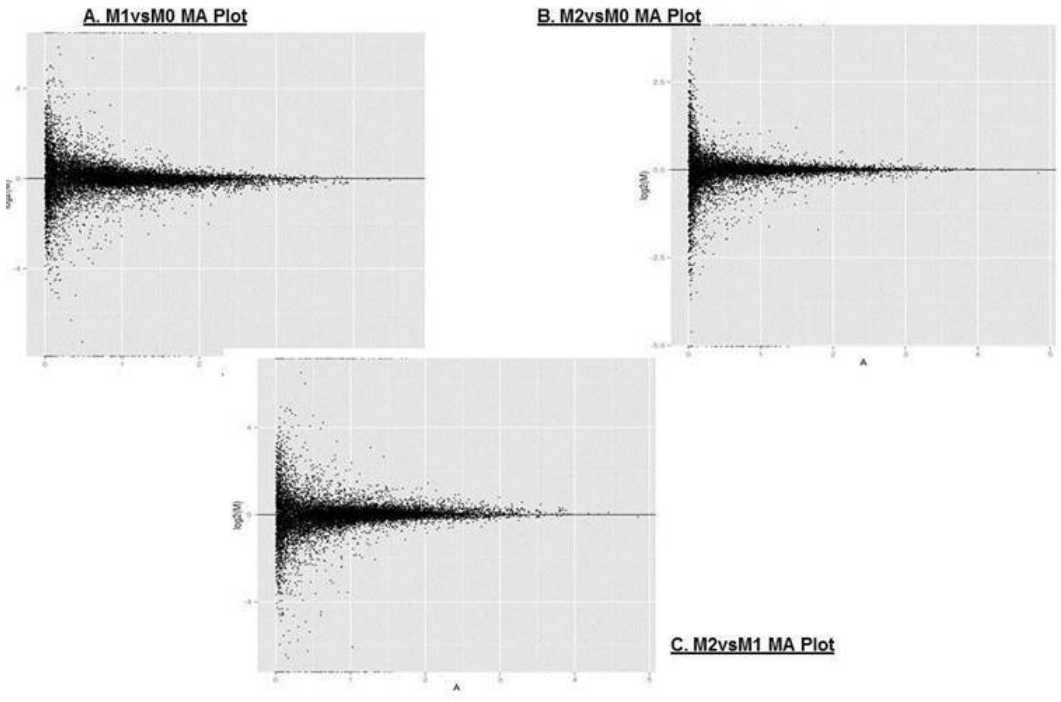

Figure 3.4: MA Plot Visualizing the Values of M1, M2 and M0 Genes Compared to Each Other.Log10(M1/M0) indicates the fold-changes in the abundances of annotated genes between the two samples; (log2(M0)+log2(M1))/2 shows the average abundance of an annotated genes.

Together, these analyses demonstrate the deviation in individual transcript expression levels, which is probably due to a great number of low abundance transcripts in all of the samples, an occurrence generally witnessed in RNA sequencing data studies.

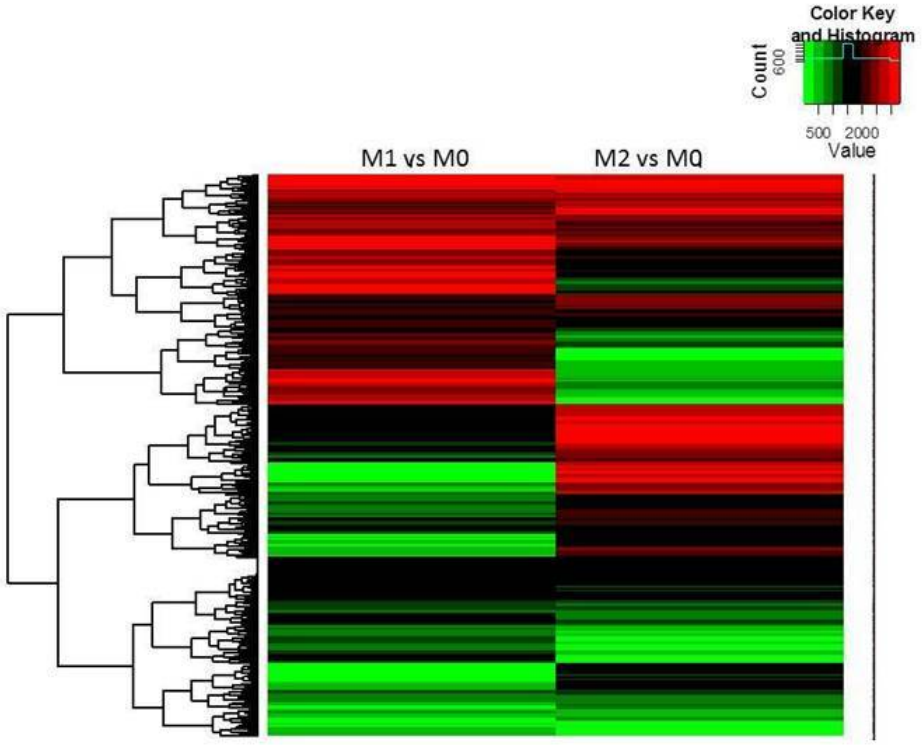

Figure 3.5: Cluster Analysis of Differentially Expressed Genes. Macrophage polarization related gene expression is summarized from profiles in M1 or M2 comparing to M0. A total of 1934 genes were upregulated and 1664 downregulated in M1 vs M2.

3.2 M1 Vs M2 Macrophages Have More Number of Differentially Expressed Genes

To estimate the differentially expressed genes and transcripts between the three macrophage groups, we set fold change of 2 as a lower bound for our subsequent analyses [Fig 3.5] [Table 3.4]. We identified 3010 transcripts that were differentially expressed between the M1 and M0 groups. Among the identified transcripts, 1556 transcripts exhibited a higher abundance (up-regulated) in the M1 group, and 1454 manifested lower relative abundance (down-regulated) than in the M0. When the M2 group was compared with the M0 group, 1011 transcripts were identified as

differentially expressed. Among these transcripts, 611 were up-regulated, and 400 down-regulated in M2. The M1 and the M2 group comparison revealed 2256 transcripts that were differentially expressed and 1027 transcripts were up-regulated and 1229 down-regulated in M1.

	M1 vs M0	M2 vs M0	M1 Vs M2
Genes differentially expressed (log2 fold change >1)	1803	765	1934
Genes differentially expressed (log2 fold change <-1)	1612	521	1664

Table 3.4: Differentially Expressed Genes Among M0, M1 and M2.Genes are considered differentially expressed with log2 fold change value greater than 1 or less than -1. It is found that there are around 1300 differentially expressed genes in M2 compared to M0. 2400 genes were differentially expressed in M1 vs M0. About 1900 genes were highly expressed in M1 compared to M2 and around 1600 genes were under expressed in M1 compared to M2.

The comparative analyses of these three samples at gene level [Fig 3.6] provide a complete view of the whole transcriptomic changes among the three groups. To further explore the pairwise relationship, regression plots were constructed which explain that M2vsM0 have R-Square value of 0.95 when compared to 0.90 and 0.91 for M1vsM0 and M1vsM2 plots [Fig 3.7].

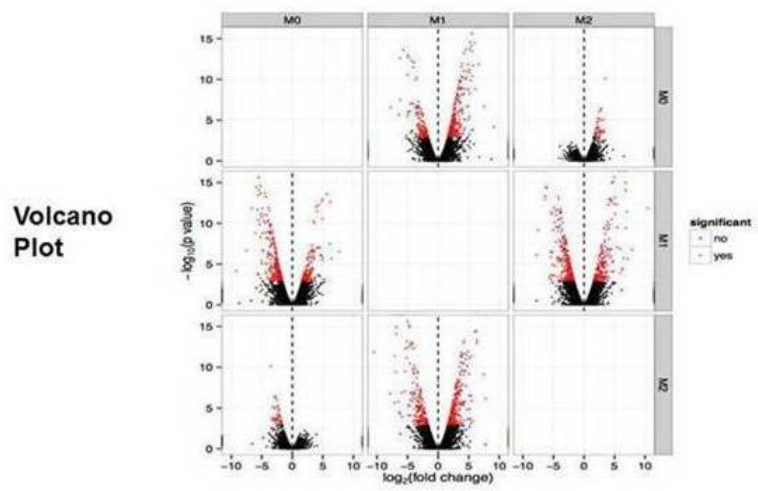

Figure 3.6: Differentially Expressed Genes Among M0, M1 and M2 Macrophages. Shown is a Volcano plot comparing the normalized expression of gene probe sets of macrophage polarization. Each red dot indicates a gene with more than 2-fold change and p-value less than 0.01 comparing to M0. Macrophage polarization related gene expression is summarized from profiles in M1 or M2 comparing to M0. A total of 1934 genes were up-regulated and 1664 down-regulated in M1 vs M2.

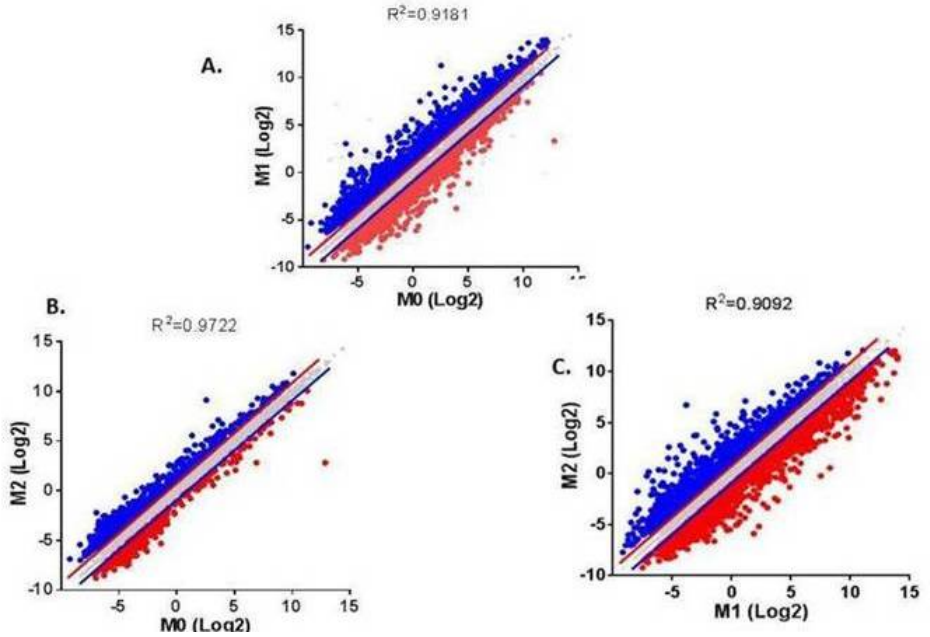

Figure 3.7: Regression Profiles Signify That M2 and M0 Macrophages Have a Very High R Square Values

There are plenty of mRNAs that are differentially expressed between M1 and M2 when compared to M0 [Fig 3.8 and Table 3.5]. Certain genes related to Antigen presentation and Processing, P53 signaling pathways, pro-inflammatory pathways have found to be highly expressed in M1 compared to M2. Whereas genes that a play role in anti-inflammatory pathways, focal adhesion and Extracellular Matrix (ECM) interaction pathways were abundantly expressed in M2 samples. This was much evidently proved by Gene Ontology (GO) studies as well.

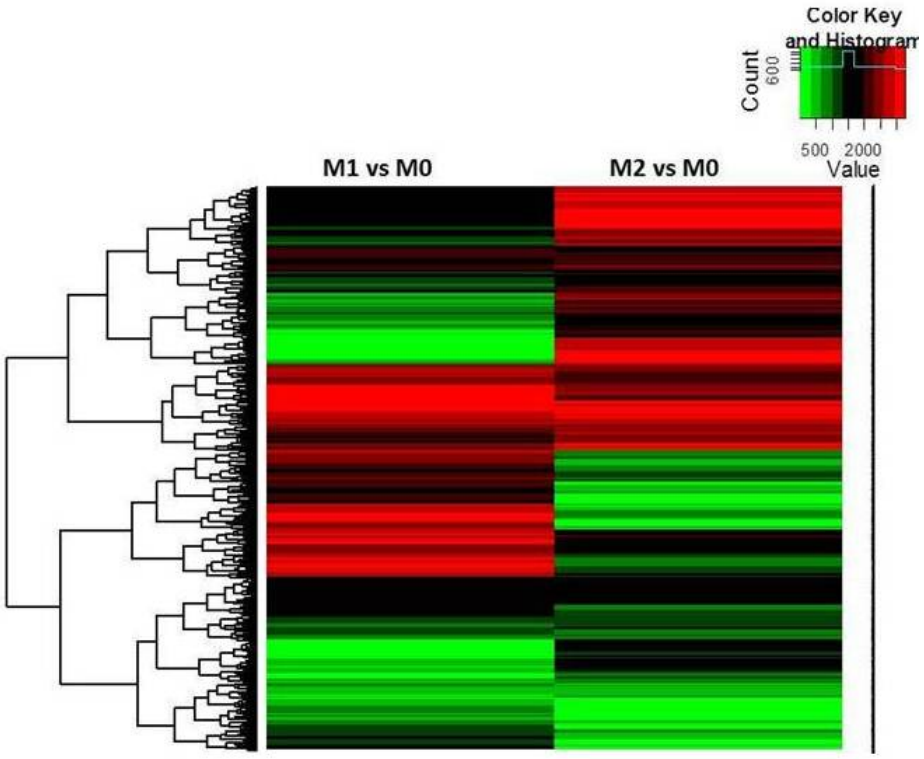

Figure 3.8: Cluster Analysis of Differentially Expressed mRNAs. Macrophage polarization related mRNAs expression is summarized from profiles in M1 or M2 comparing to M0. Several mRNAs are distinctly differentially expressed among the two groups.

Moreover, the differential expression of transcripts that resulted in more than one assessment were separated from the bona-fide transcripts that were specific to one comparison, i.e., the transcripts specific for M1 vs. M0 (3413 genes, 3010 mRNAs), M2 vs. M0 (1011 mRNAs, 1286 genes) and M1 vs. M2 (2256 mRNAs, 3598 genes) [Fig 3.9 and Table 3.6].

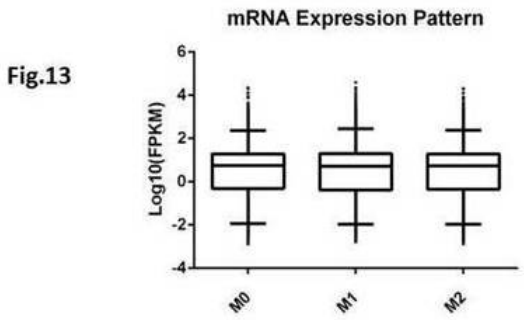

Figure 3.9: Analysis of Protein Coding Genes. Box plot showing distribution of the expression profiles of the three differently activated macrophages. Each box is based on 24000 genes based on their FPKM values. Median FPKM values are denoted by black solid lines and the top and bottom box edges denote the first and third quartile of FPKM values comprising genes. Whiskers represents the largest and smallest data within 1.5 times that of the interquartile range of FPKM.

	M0	M1	M2
No. of Genes Expressed	14353	14389	14358
No. Protein coding genes	11598	11594	11725

Table 3.5: Analysis of Protein Coding Genes.Most of the genes expressed are protein coding genes.

	M1 vs M0	M2 vs M0	M1 Vs M2
mRNA differentially expressed (log2 fold change >1)	1556	611	1027
mRNA differentially expressed (log2 fold change <-1)	1454	400	1229

Table 3.6: Differential Expression Pattern of mRNAs: It is found that there are around1000 differentially expressed mRNAs in M2 compared to M0. 2000 mRNAs were differentially expressed in M1 vs M0. About 1027 mRNAs were highly expressed in M1 compared to M2 and around 1229 mRNAs were under expressed in M1 compared to M2.

Interestingly, when we compared the abundantly expressed genes in all M0, M1 and M2 (FPKMM$\geq$100), it was revealed that 703 transcripts were highly expressed in all three samples.

It signifies that these genes could be responsible for the basic physiological functions of all the macrophages. 245 genes were abundantly expressed in M1 alone compared to 54 and 22 in M2 and M0 [Fig 3.10].

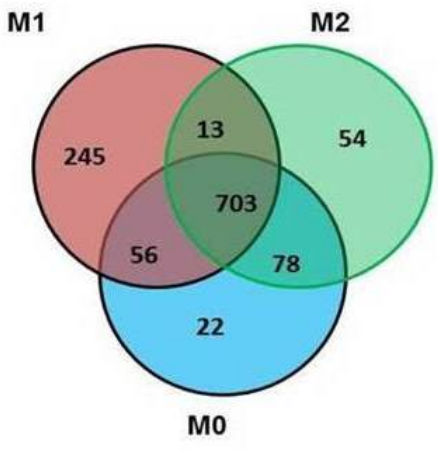

Figure 3.10: Genes Expressed More than 100 FPKM. Three sets of macrophage profiles comparing M1, M2 and M0 samples were analyzed to identify genes which are consistently upregulated (FPKM $\geq$ 100). Analysis revealed that 703 genes are highly expressed (FPKM $\geq$ 100) in all M0, M1 and M2 samples and 22, 245 and 54 are uniquely highly expressed (FPKM $\geq$ 100) in M0, M1 and M2.

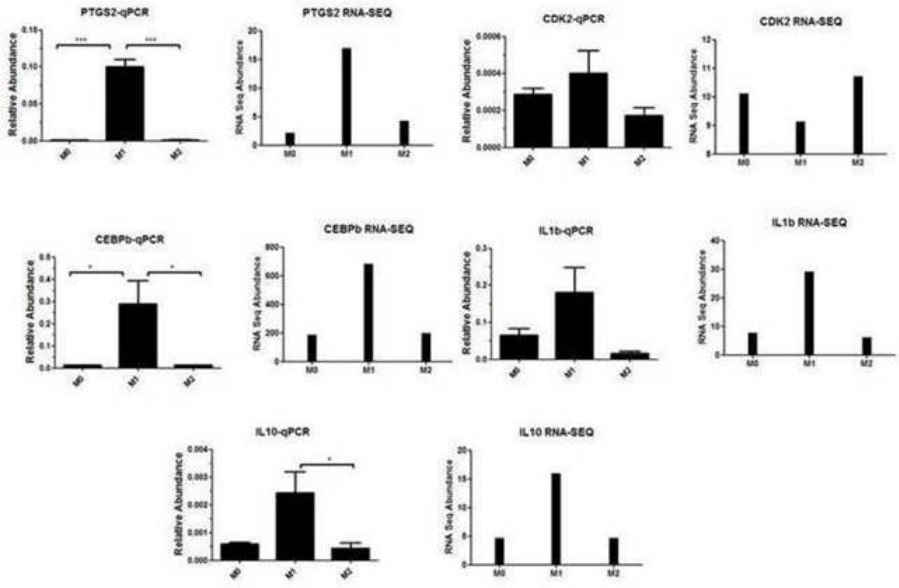

Figure 3.11: Validation of RNA Seq Data for Certain Genes Using qPCR. Expression levels of PTGS2 was found to be significantly higher in m1 compared to M0 and M2. Il10, IL1b, CDK2 and CEBPb all follow the similar expression pattern as the data sets of RNA-seq revealed.

We further did a qPCR analysis of certain transcripts in all the three groups M0, M1 and M2. These observations indicate the accuracy of the RNA sequencing-based transcript reconstruction and abundance calculation (Fig 3.11).

3.3 Crosstalk Between Signaling Pathways

To associate cellular functions with the set of differentially expressed genes, we used DAVID Gene Ontology program and Ingenuity pathway analysis. Gene networks were assessed using the IPA library of canonical pathways. Specifically for transcriptional data, the genes that were differentially expressed with at least 2-fold enriched occupancy of M1 and M2 compared to M0 were associated with canonical pathways using the Ingenuity Knowledge Base. Using this approach, the top canonical pathways that were found to be affected by the genes which are highly expressed in M1 compared to M0 are Dendritic cell Maturation, Graft versus Host Disease Signaling, Trem1 signaling and Type 1 Diabetes Mellitus [Fig 3.12].

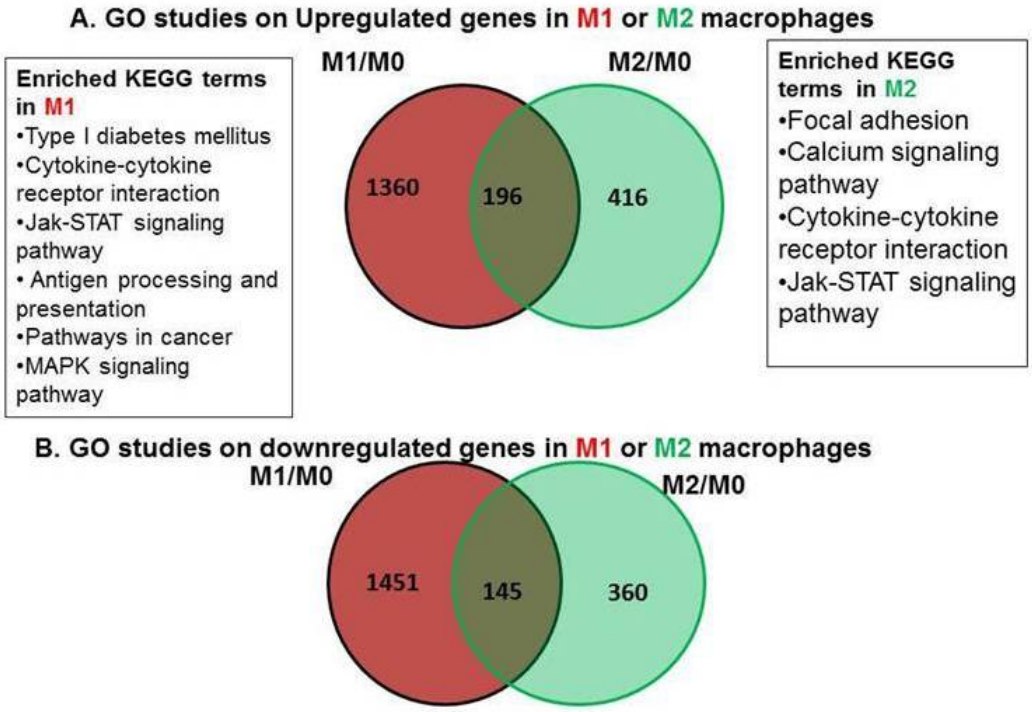

Figure 3.12: Gene Ontology analysis for Genes with More than 2 Folds Alteration During Macrophage Polarization. Cellular functions of significantly altered genes in M1 or M2 are analyzed. In M1 macrophages, several GO terms are significant enriched ($P \leq 0.001$) including Type I Diabetes mellitus and Antigen Presenting and Processing; whereas ECM receptor interaction is highly enriched in M2

This is noteworthy, because these canonical pathways are involved in all aspects of known M1 macrophages. In upstream regulator analyses, we found that MYD88 (Myeloid differentiation Primary response 88), TICAM1 (Toll - Like Receptor Adaptor Molecule1), IFNG, SOCS1 (Suppressor of Cytokine Signaling1) and IFNB1 are the top regulators in M1 compared to M0 [Table 3.7a,b and c]. Genes highly expressed in M1 datasets are also involved in some of the important disease and disorders including infectious disease, immunological disease, and gastrointestinal disease to name few. We next identified those biological functions most significantly asso-

ciated with the M1 RNA-Seq datasets using the IPA Functional Analysis feature.

A. Top Upstream Regulators

Upstream Regulators in M1	P value
IFNG	1.89E-59
TICAM1	1.93E-56
STAT1	1.59E-53
TLR4	7.47E-53
MYD88	1.54E-46

B. Top Canonical Pathway

Name	p-value	Ratio
Dendritic Cell Maturation	2.35E-18	49/173 (0.283)
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	1.17E-17	34/84 (0.405)
TREM1 Signaling	9.34E-13	23/54 (0.426)
Graft-versus-Host Disease Signaling	1.05E-12	20/42 (0.476)
Type I Diabetes Mellitus Signaling	2.12E-12	32/109 (0.294)

C. Diseases and Disorders

M1	P-Value	No. of molecules
Immunological Disease	1.15E-52 - 1.03E-05	227
Endocrine System Disorders	6.17E-42 - 2.83E-07	134
Gastrointestinal Disease	6.17E-42 - 9.86E-06	200
Metabolic Disease	6.17E-42 - 1.46E-36	164
Infectious Disease	6.39E-32 - 7.91E-06	151

Table 3.7: M1 IPA Analyses. IPA analyses of the genes expressed in M1 showed that TLR4, IFNg, Stat1 are some of the top upstream regulators for the different functional pathways the genes expressed in M1 are involved. IFNg is found to play role in antigen presentation and processing, allograft rejection signaling pathway and these pathways found their position in the top KEGG terms. Right-tailed Fishers exact test was used to calculate p-values determining the probability that each biological function was due to chance alone.

This highlighted that inflammatory response genes were the top biological function comprising of about 109 genes. David Gene Ontology studies revealed that genes which are highly expressed in M1 when compared to M0 enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) terms like Type I Diabetes mellitus, Antigen Presentation and Processing, Pathways in cancer to highlight few. On the other

hand, KEGG terms enriched by genes highly expressed in M2 when compared to M0 include Focal adhesion, calcium signaling pathway, and ECM receptor interaction to name some [Fig 3.13].

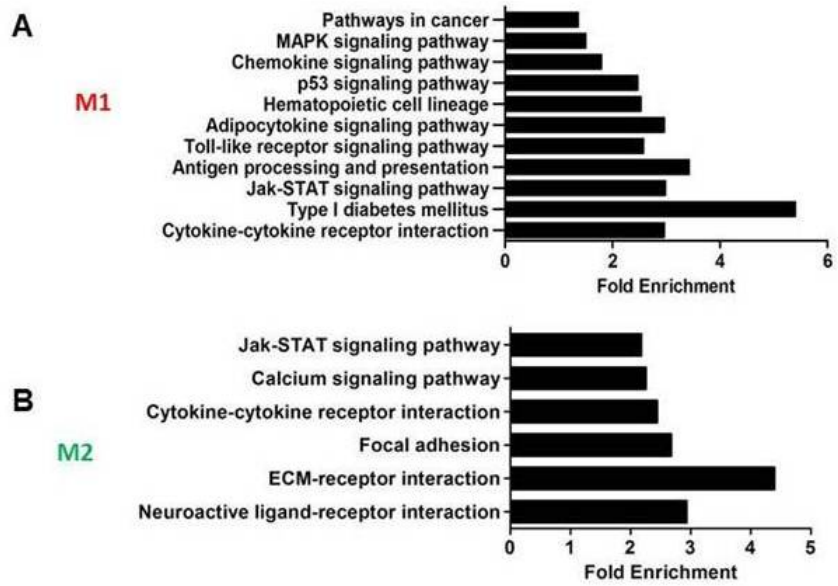

Figure 3.13: Significantly Enriched GO Terms in M1 or M2 Activation. The fold enrichment of the KEGG terms was analysed and was found that with the genes differentially expressed in M1 some of the key KEGG terms with high fold enrichment includes Antigen Presentation and Processing, Type I Diabetes mellitus, Adipocytokine signaling pathway and others. Whereas the differentially expressed genes in M2 are found to have KEGG terms with high fold enrichment like ECM-receptor interaction, Calcium Signaling and others.

Using IPA, when M2 datasets were analyzed, it revealed that miR-223 is one of the top upstream regulators among other significant regulators like IL4, IGF1r (Insulin Growth Factor 1 receptor), CCR2 (Chemokine (C-C Motif) Receptor 2) and others [Table 3.8a].

Role of miR-223 was significantly proved in previous research studies of our lab

A. Top Upstream Regulators

Upstream Regulators	P value
miR-223	7.64E-09
TP53	2.27E-07
IL4	6.23E-25
IGF1R	8.95E-03
CCr2	8.37E-03

B. Top Canonical Pathway

Name	p-value	Ratio
Protein Ubiquitination Pathway	4.54E-28	229/264
B Cell Receptor Signaling	1.17E-20	146/165
Glucocorticoid Receptor Signaling	1.42E-20	222/279
Molecular Mechanisms of Cancer	5.14E-20	278/368
Huntington's Disease Signaling	5.33E-18	187/232

C. Diseases and Disorder

Names	P-Value	No. of molecules
Infectious Disease	2.92E-66 - 5.29E-11	1742
Cancer	1.16E-28 - 1.38E-10	2259
Organismal Injury and Abnormalities	2.90E-22 - 6.94E-11	1244
Immunological Disease	7.62E-22 - 7.76E-11	1698
Hematological Disease	9.32E-22 - 1.12E-10	1297

Table 3.8: M2 IPA Analyses.IPA analyses of the genes expressed in M2 showed that miR223, IL4, IGF1r are some of the top upstream regulators for the different functional pathways the genes expressed in M2 are involved. B cell receptor signaling, protein ubiquitination pathway, molecular mechanisms of cancer found their position in the top KEGG terms. Right-tailed Fishers exact test was used to calculate p-values determining the probability that each biological function was due to chance alone.

[28]. Mir-223 is known to up-regulate expression of Ataxia Telangiectasia, NFIA (Nuclear factor I/A), SLC2A4 (Solute Carrier Family 2, member 4) and others. It is also found to down-regulate genes including STAT3 (Signal Transducer and Activator of Transcription 3), FBXW7, IGF1R and many more. Interesting knock down mir-223 studies showed that it also reduces the expression levels of IGF1, TLR13 (Toll Like Receptor13), and TGFb1 to name some [Fig 3.14].

Studies on mir-223 have shown that it inhibits nuclear translocation of STAT3 in glomerular endothelial cells. STAT3 is a very important transcription factor involved

in regulation of transcription of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis [73]. Genes highly expressed in M2 datasets are found to be involved in different canonical pathways including B cell receptor signaling, Glucocorticoid signaling, Molecular mechanisms of cancer and lot more[Table 3.8b]. Macrophages activate to M2 status are also involved in different disease and disorder conditions including cancer, infectious disease, immunological disease, hematological disease and many more[Table 3.8c].

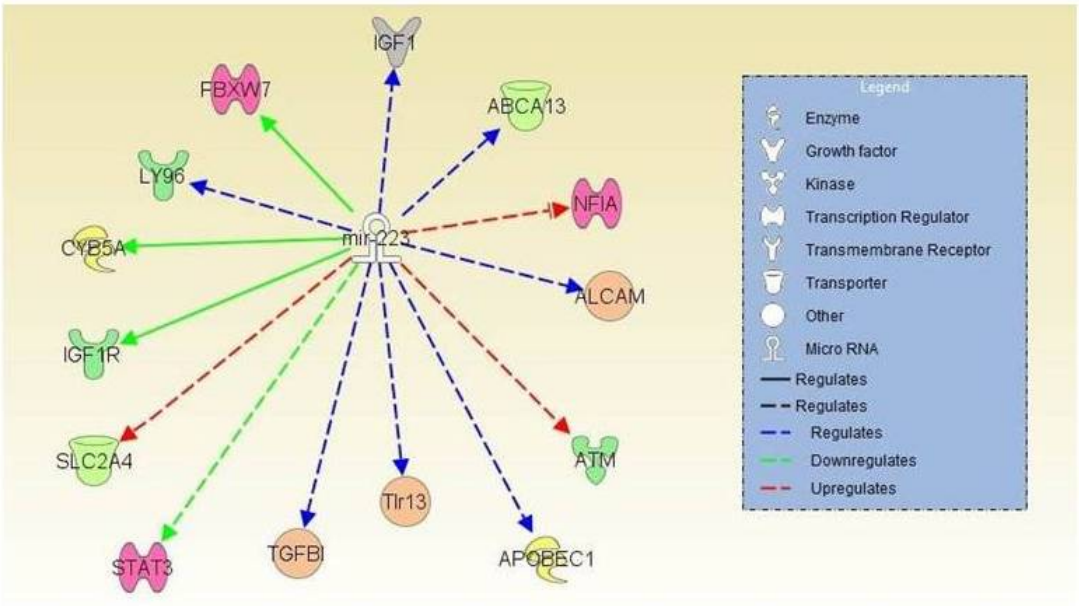

Figure 3.14: MiR223 Regulates Different Genes Which Impact Various Functional Pathways. miR223 decreases the expression of STAT3 and IGF1r. STAT3 is a very important transcription factor involved in regulation of transcription of a range of genes in reaction to cell stimuli, and thus is involved in many cellular developments such as cell growth and apoptosis. Knockdown of miR-223 decreased expression of Tlr13, TGFb1, IGF1, Ly96 and others highlighted with blue arrow.

3.4 Role of M1 in Diabetes Type I Mellitus is Signified by High Expression of PPARG and IGF2bp2

IPA analysis revealed that classically activated macrophages have genes with high expression that are involved in Diabetes mellitus. Some of the notable genes are Pparg (Peroxisome Proliferator-Activated Receptor),FTO (Fat Mass and Obesity Associated),Kcnj11 (Potassium Inwardly-Rectifying Channel, Subfamily J, Member 11), Notch2,Wfs1 (Wolfram Syndrome 1),Igf2bp2,Slc30a8,Jazf1 (Jazf Zinc Finger 1),Hhex (Hematopoietically Expressed Homeobox),Cdkal1,Cdkn2a,Capn10 (Calpatin 10) and the research team of Shukui Wang identified two additional genes Rapgef(Rap Guanine Nucleotide Exchange Factor) andTp53 (Tumor protein 53) related to type 2 diabetes risk. The authors suggest that Rapgefrepresents a strong candidate because of its role in insulin signaling. In addition, theRapgefpathway may be involved in the regulation of proglucagon gene expression in intestinal endocrine L-cells [74], providing another mechanism for its effect on risk of type 2 diabetes. Rapgef is very highly expressed in M1 when compared to M2 and M0. Other genes like Pparg and Igf2bp2 are also very highly expressed in M1. Pparg is very well known for its involvement in inducing adipogenesis and its over expression also increases insulin sensitivity that is of great benefit in diabetes treatments [75]. Igf2bp2 is insulin like growth factor-2 protein which play a vital role in the regulation of Insulin Growth Factor2 expression. IGF2B2 functions by binding to 5'UTR of the IGF2 mRNA and regulates IGF2 translation.

3.5 High Expression of CIITA and TAP Genes Shows Role of M1 in Antigen Presentation Pathway

Of the many different genes that are involved in antigen presentation pathway, Some genes like CD74, CIITA (Class II, Major Histocompatibility Complex, Trans-

activator), IFNG, PSM8 and 9, TAP1 (Transporter 1, ATP-Binding Cassette) and 2 and TAPBP are very highly expressed. CD74 is related with class II major histocompatibility complex (MHC) and is an significant chaperone that controls antigen presentation for immune response [76]. Studies also revealed that It serves as cell surface receptor for the cytokine macrophage migration inhibitory factor (MIF) which initiates survival pathways and cell proliferation when bound to the encoded protein [77]. TAP 1 and 2 are the one that hold the peptide fragments and transport it to the endoplasmic reticulum from the cytoplasm [74]. CIITA is responsible for activation of MHC1 and II to which CLIP protein binds and aids in the transport of peptide fragment from ER to Golgi and then to late endosome. IFNG is very much involved in activation of TAP1 and 2 and making them bind to the peptide fragments. It also activates CIITA and NLRC5, which are involved in transport of peptide fragments [78]. The expression levels of these genes in M1 signified their role in antigen presentation. TAP1 and TAP2 expression levels are significantly higher in M1 with FPKM about 331 and 343 compared to 146.1 and 94.8 in M0 respectively. High CD74 expression levels of 2228.3 in M1 compared to 713.29 in M0 reveals that classically activated macrophages are involved in antigen presentation and processing.

3.6 Genes Related to Atherosclerosis Signaling are Highly Expressed in M2

IPA analysis revealed that alternatively activated macrophages have genes with high expression that are involved in atherosclerosis signaling. Some of the notable genes are involved in diabetic atherosclerosis are Ager, collagen, MMP2 (Matrix Metallopeptidase2), MMP9, NFKB1 (Nuclear Factor of kappa light polypeptide gene enhancer in B-cells), PTGS2 and RELA. Of these, MMP9, NFKB1 and PTGS2 genes are very highly expressed signifying the role of alternate activated macrophages in atherosclerosis. In atherosclerosis signaling, triggering of CCR2 by Low Density

Lipoprotein (LDL) leads to monocyte-endothelial cell adhesion via ICAM-1 and VCAM-1. MCP1 mediated transendothelial migration of monocytes into intima is followed [79]. M-CSF produced by endothelial cells differentiates monocytes to macrophages. In these macrophages, uptake of oxidized LDL via scavenger receptors leads to lipid accumulation and foam cell formation. In foam cells, the tumor necrosis factor receptor is activated by a ligand, which increases the expression of IL6, IL8, MCP1 and NFKB1 to further activate MMP9 [80]. MMP proteins act on the collagen released from the smooth muscle cells. M2 macrophages are also very much involved in anti-inflammatory signaling and other processes.

3.7 Regulation of Expression by IFN-Gamma Play Important Role in Allograft Rejection and Antigen Presentation

IFN-gamma plays a significant role in different functional roles. It binds to IFNGR1, STAT receptors, JUN, CREB (Camp Responsive Element Binding Protein), NFAT (Nuclear Factor Of Activated T-Cells) and other, and regulates certain genes like NOS2, IRF1, STAT1, CXCL10, TNF, ICAM1, MHC classII which are involved in some of the important functional pathways involving antigen presenting pathway, atherosclerosis signaling, allograft rejection signaling and others [Fig 3.15].

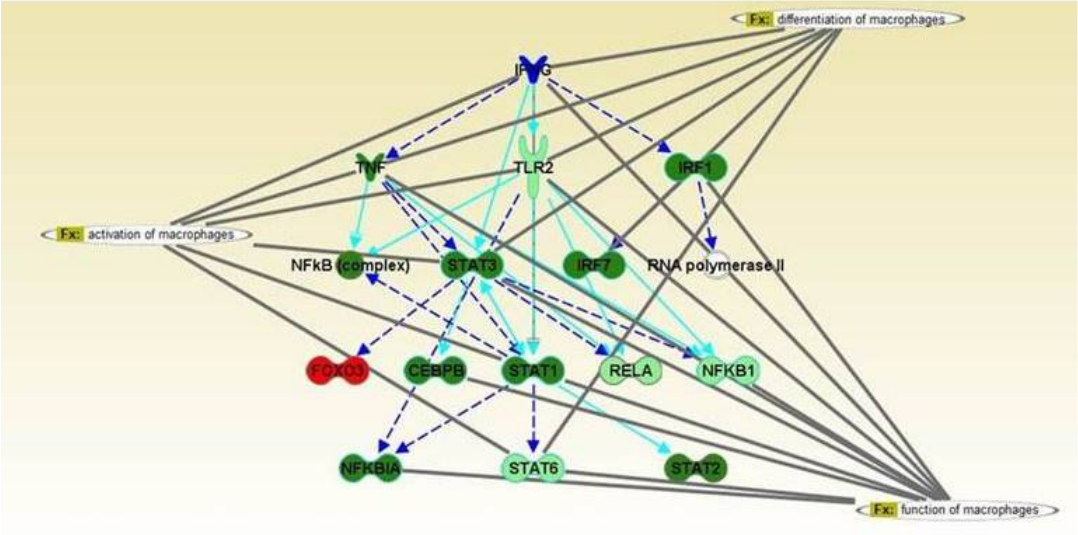

Figure 3.15: IFNg- One of the Top Upstream Regulator. IFNg regulates expression of several genes and further impact their role in different functional pathways. IFNg plays a pivotal role in regulation of NFkB, Stat and TNF contributing to its role in activation of macrophages. Some of the genes which are involved in differentiation of macrophages are also regulated by IFNg.

In allograft rejection signaling pathway, IFN-G stimulates macrophages to produce NO and TNF-alpha, which play a role in cytotoxicity towards the donor cell [81]. In our datasets, TNF expression levels in M1 macrophages (FPKM= 94.83) are significantly higher when compared to the expression levels in M1 and M0 (FPKM 7.4 and 8.83). In antigen presentation pathway, IFN-G stimulates activation of CIIA (MHC co-activator) and NLRC5 which further stimulates the MHCII to bind to the peptide fragment and MHCI bind to the CLIP (Cap-Gly Domain Containing Linker Protein) (CD74 MHCII variant) [77]. Supporting this the datasets also show expression levels of CIITA found FPKM value to be around 10.825 for M1 whereas in M2 and M0 it is expressed around 6.26 and 3.28 respectively. Also, NLRC5 expression value is 87.27 whereas the expression levels in M2 and M0 are very less about 27.88 and 30.28 respectively.

3.8 LPS Induces Genes Responsible for Cellular Immune and Humoral Response

LPS is an endotoxin protein and binds to certain receptors including TLR4, CD14, APCS, ITGB2 and others and induces different immune related functional pathways [74]. Binding of LPS to TLR stimulate production of IFN-Beta that play a role in adaptive immune response [82]. Our dataset also clearly shows that the expression of IFN-Beta was significantly enhanced to 29.97 in M1 when compared to 0.69 FPKM of M0. In response to LPS, TLR also stimulates IL8 and IP10, which further took part in chemo attraction of neutrophils, TH1 cells and monocytes [82]. In addition, TLR also enhances MIP1 production that is involved in the chemoattraction of T cells and dendritic cells. IL12 synthesis is also increased which further enhances IL10 and IFNG, which further play a role in the development of humoral immune response and development of cellular immune response respectively [83]. The enhanced expression levels of IL10 and IFNG is also revealed in our datasets showing expression levels of FPKM 15.94 and 13.67 in M1 compared to 4.63 and 2.42 in M0 respectively. LPS also activates P38 MAPK and JNK through TLR that together enhance IL12-P40 playing pivotal role in signaling and production of macrophages. IL12-P40 further activates Il12-P80 which has roles in TH1 activation and inhibition, macrophage recruitment and DTH [84]. LPS also has role in the production of nitric oxide and reactive oxygen species. It triggers MIK and PI3K expression which in turn play a role in the production of nitric oxide that is important for protection against infection and neoplasia and inflammation ageing neurotoxicity [76]. Activation of Reactive oxygen species plays a role in innate immune response against microbes.

3.9 IL4 Aids in THh1 Response

There are certain pathways and genes that are being induced by Interleukin-4. IL4 is a very well-studied TH2 cytokine that is involved in induction and initiation

of various functional pathways. IL4 binds to IL4R, IL2RG, Nfat, STAT6 and others. These binding in turn regulate certain functional pathways including allograft rejection, T helper cell differentiation, hematopoiesis from pluripotent stem cells and other important functional roles. In allograft rejection signaling, IL4 induces B cells which are bound by MHCII and CD40 which further aids in alloantibody graft rejection by producing IgG which further plays role in Complement aided damage by action on MHCII [85]. In crosstalk between dendritic and natural killer cell, IL4 triggers the expression of DAP12 and TREM2 (Triggering Receptor Expressed On Myeloid Cells 2). Trem2 further regulates the action of IFN-G [77]. In IL12 signaling, IL4 induces STAT6 that will further inhibit the action of STAT4 on IFN-G that is playing important role in TH1 response in intracellular bacterial death [79].IL4 also has an important part to play in hematopoiesis from pluripotent stem cells.

3.10 Genes and Pathways Induced Through IL13

IL13 induces many important functional pathways. It binds to IL13RA1, IL4R, STAT6, CHD4, AP1 and others. It regulates ARG1, TNF, NOS2, TGFB1, STAT6, CCL4 and these will further regulate crucial biological functions including differential regulation of Cytokine production in Macrophages and T Helper cells by IL17, T Helper cell differentiation, Role of cytokines in mediating communication between immune cells.

3.11 Interaction Between IL4, IL13 and IFNG

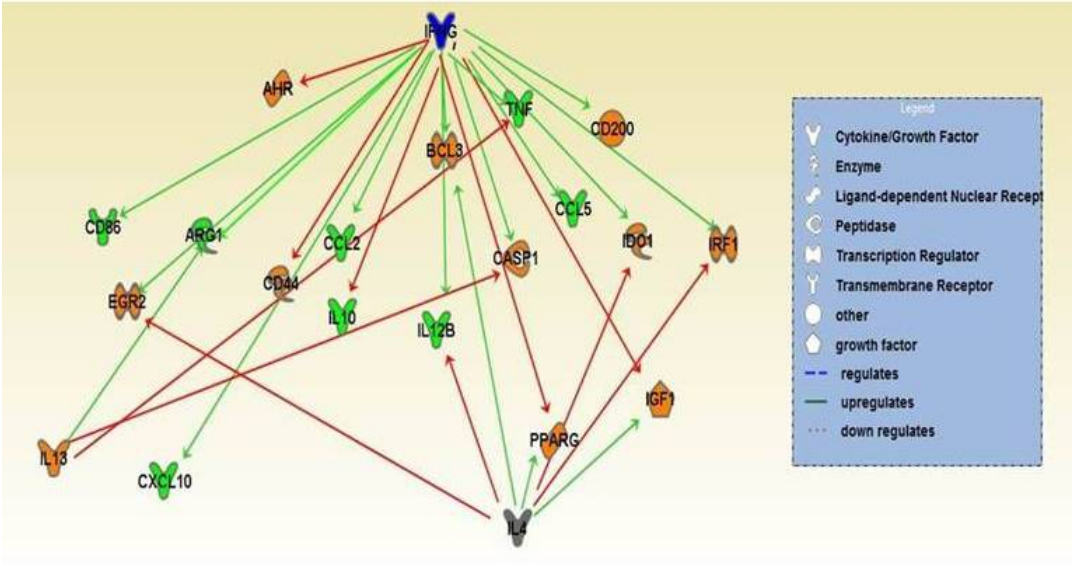

Figure 3.16: IFNG, IL4 and IL13 Interaction Pattern. IFNG, IL4 and IL13 have different impacts on different genes. IL13 increases the expression of CASP1 whereas IFNg down-regulates its expression. Casp1 play pivotal role in apoptosis. IFNg decreases the expression levels of IRF1 but IL4 increases its expression. Ifng decreases the expression of insulin growth factor 1 (IGF1) and also do have an effect on the reducing effect of insulin. Il4 induction of macrophages induces the expression of IGF1, correlating its effect on enhancing insulin sensitivity. Ifng stimulates the expression of Interferon regulatory factor1 .

IL4 upregulates Pparg whereas Ifng downregulates it. Pparg that plays an important role in M2 activation and also in different physiological function in which M2 is involved are regulated through Il4 and Pparg as well. Ifng decreases the expression of insulin growth factor 1 (IGF1) and also do have an effect on the reducing effect of insulin. Il4 induction of macrophages induces the expression of IGF1, correlating its effect on enhancing insulin sensitivity. Ifng stimulates the expression of Interferon regulatory factor1 [Fig 3.16].

4. CONCLUSION AND FUTURE PROSPECTS

In summary, we have documented for the first time the gene expression profiles for the macrophages in different activation states by correlating our data with qPCR. Globally, approximately 3000 genes were differentially expressed among the M0, M1 and M2 states of macrophages. With regard to differentially expressed genes, a high correlation was observed between M0 and M2 in RNA-Seq data. Gene ontology analysis indicated abundantly expressed genes in M1 significantly impacted Type I diabetes and antigen presentation and processing. On the other hand, focal adhesion, ECM interaction signaling are influenced by genes highly expressed in M2. Interestingly IFNG plays important role in functions related both to M1 and M2. In conclusion, distinct combinatorial patterns of gene expression exist in classically and alternate activated macrophages. These specific differences may explain the differential effects of macrophages in its different roles. Future research could be a profound understanding of the complex network of interactions among different factors involved in state of polarization of macrophages in health and disease.

REFERENCES

[1] M K Ohman, Y Shen, C I Obimba, A P Wright, M Warnock, D A Lawrence, and D T Eitzman. Visceral adipose tissue inflammation accelerates atherosclerosis in apolipoprotein E-deficient mice. *Circulation*, 117(6):798–805, 2008.

[2] A H Berg and P E Scherer. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res*, 96(9):939–949, 2005.

[3] G S Hotamisligil, P Arner, J F Caro, R L Atkinson, and B M Spiegelman. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest*, 95(5):2409–2415, 1994.

[4] F Masato, F Raquel, Z G Cem, T Grol, C Haiming, and S H Gkhan. Adipocyte/macrophage fatty acidbinding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest*, 118(7):2640–2650, 2008.

[5] S P Weisberg, D McCann, M Desai, M Rosenbaum, R L Leibel, and A W Jr. Ferrante. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*, 112(12):1796–1808, 2003.

[6] C N Lumeng, J L Bodzin, and A R Saltiel. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest*, 117(1):175–184, 2007.

[7] K Kang, S M Reilly, V Karabacak, M R Gangl, K Fitzgerald, B Hatano, and C H Lee. Adipocyte-derived Th2 cytokines and myeloid PPAR delta regulate macrophage polarization and insulin sensitivity. *Cell Metab*, 7(6):485–495, 2008.

[8] Z X Meng, G X Wang, and J D Lin. A microRNA circuitry links macrophage polarization to metabolic homeostasis. *Circulation*, 125(23):2815–2817, 2012.

[9] J M Olefsky and C K Glass. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol*, 72:219–246, 2010.

[10] S K Biswas and A Mantovani. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*, 11(10):889–896, 2010.

[11] J C Rodrguez-Prados, P G Travs, J Cuenca, D Rico, J Aragons, P Martn-Sanz, M Cascante, and L Bosc. Substrate fate in activated macrophages: A comparison between innate, classic, and alternative activation. *J Immunol*, 185(1):605–614, 2010.

[12] H F Lodish, B Zhou, G Liu, and C Z Chen. Micromanagement of the immune system by microRNAs. *Nat Rev Immunol*, 8(2):120–130, 2008.

[13] D Baltimore, M P Boldin, R M O'Connell, D S Rao, and K D Taganov. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol*, 9(8):839–845, 2008.

[14] S Gordon and P R Taylor. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*, 5(12):953–964, 2005.

[15] D M Mosser and J P Edwards. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*, 8(12):958–69, 2008.

[16] J I Odegaard and A Chawla. Mechanisms of macrophage activation in obesity-induced insulin resistance. *Nat Clin Pract End Met*, 4(11):619–626, 2008.

[17] C N Lumeng, S M Deyoung, and A R Saltiel. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am J Physiol-Endoc M*, 292(1):E166–E174, 2007.

[18] F O Martinez, S Gordon, M Locati, and A Mantovani. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol*, 177(10):7303–7311, 2006.

[19] A Mantovani, A Sica, S Sozzani, P Allavena, A Vecchi, and M Locati. The chemokine system in diverse forms of macrophage activation and polarization. *Trend Immunol*, 25(12):677–686, 2004.

[20] C Porta, M Rimoldi, G Raes, L Brys, P Ghezzi, D Di Liberto, F Dieli, S Ghisletti, G Natoli, P De Baetselier, A Mantovani, and A Sica. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB. *Proc Natl Acad Sci U S A*, 106(35):14978–14983, 2009.

[21] U Yamashita and E Kuroda. Regulation of macrophage-derived chemokine (MDC, CCL22) production. *Crit Rev Immunol*, 22(2):105–114, 2002.

[22] M Kurowska-Stolarska, B Stolarski, P Kewin, G Murphy, C J Corrigan, S Ying, N Pitman, A Mirchandani, B Rana, N van Rooijen, M Shepherd, C McSharry, I B McInnes, D Xu, and F Y Liew. Il-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol*, 183(10):6469–6477, 2009.

[23] R M Tighe, J Liang, N Liu, Y Jung, D Jiang, M D Gunn, and P W Noble. Recruited exudative macrophages selectively produce CXCL10 after noninfectious lung injury. *Am J Respir Cell Mol Biol*, 45(4):781–788, 2011.

[24] P Loke, M G Nair, J Parkinson, D Guiliano, M Blaxter, and J E Allen. Il-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol*, 3:7, 2002.

[25] S K Biswas, A Sica, and C E Lewis. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol*, 180(4):2011–2017, 2008.

[26] A Mantovani and M Locati. Orchestration of macrophage polarization. *Blood*, 114(15):3135–3136, 2009.

[27] C N Lumeng, S M Deyoung, J L Bodzin, and A R Saltiel. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes*, 56(1):16–23, 2007.

[28] G Zhuang, C Meng, X Guo, P S Cheruku, L Shi, H Xu, H Li, G Wang, A R Evans, S Safe, C Wu, and B Zhou. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. *Circulation*, 125(23):2892–2903, 2012.

[29] S Banerjee, N Xie, H Cui, Z Tan, S Yang, M Icyuz, E Abraham, and G Liu. MicroRNA let-7c regulates macrophage polarization. *J Immunol*, 2013.

[30] M Hulsmans and P Holvoet. The vicious circle between oxidative stress and inflammation in atherosclerosis. *J Cell Mol Med*, 14(1-2):70–78, 2010.

[31] T Lawrence, D W Gilroy, P R Colville-Nash, and D A Willoughby. Possible new role for NF-kappa B in the resolution of inflammation. *Nat Med*, 7(12):1291–1297, 2001.

[32] C N Serhan, R Yang, K Martinod, K Kasuga, P S Pillai, T F Porter, S F Oh, and M Spite. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med*, 206(1):15–23, 2009.

[33] A Weigert, A M Johann, A von Knethen, H Schmidt, G Geisslinger, and B Brune. Apoptotic cells promote macrophage survival by releasing the an-

tiapoptotic mediator sphingosine-1-phosphate. *Blood*, 108(5):1635–1642, 2006.

[34] J K Sethi and A J Vidal-Puig. Thematic review series: adipocyte biology. adipose tissue function and plasticity orchestrate nutritional adaptation. *J Lipid Res*, 48(6):1253–1262, 2007.

[35] H Xu, G T Barnes, Q Yang, G Tan, D Yang, C J Chou, J Sole, A Nichols, J S Ross, L A Tartaglia, and H Chen. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*, 112(12):1821–1830, 2003.

[36] E W Kathryn and S H Gkhan. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest*, 112(12):1785–1788, 2003.

[37] K Clement, N Viguerie, C Poitou, C Carette, V Pelloux, C A Curat, A Sicard, S Rome, A Benis, J D Zucker, H Vidal, M Laville, G S Barsh, A Basdevant, V Stitch, R Cancello, and D Langin. Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J*, 18(14):1657–1669, 2004.

[38] J I Odegaard and A Chawla. Alternative macrophage activation and metabolism. *Annu Rev Pathol*, 6:275–297, 2011.

[39] A R Eagle and A Chawla. In obesity and weight loss, all roads lead to the mighty macrophage. *J Clin Invest*, 120(10):3437–3440, 2010.

[40] C N Lumeng, J B DelProposto, D J Westcott, and A R Saltiel. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes*, 57(12):3239–3246, 2008.

[41] V Rottiers and A M Naar. MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol*, 13(4):239–250, 2012.

[42] D P Bartel. MicroRNAs: target recognition and regulatory functions. *Cell*, 136(2):215–233, 2009.

[43] D P Bartel and C Z Chen. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet*, 5(5):396–400, 2004.

[44] D P Bartel. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, 116(2):281–297, 2004.

[45] Katey J R, J S Frederick, C E Christine, N H Farah, E T Ryan, P Saj, M G Janine, J R Alistair, N C Aaron, S Yajaira, F H Carlos, A F Edward, and J M Kathryn. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J Clin Invest*, 121(7):2921–2931, 2011.

[46] K J Rayner, Y Surez, A Dvalos, S Parathath, M L Fitzgerald, N Tamehiro, E A Fisher, K J Moore, and C Fernndez-Hernando. Mir-33 contributes to the regulation of cholesterol homeostasis. *Science*, 328(5985):1570–1573, 2010.

[47] H Xie, B Lim, and H F Lodish. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes*, 58(5):1050–1057, 2009.

[48] M Trajkovski, J Hausser, J Soutschek, B Bhat, A Akin, M Zavolan, M H Heim, and M Stoffel. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature*, 474(7353):649–653, 2011.

[49] C Ling and L Groop. Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes*, 58(12):2718–2725, 2009.

[50] Y Wei, M Nazari-Jahantigh, L Chan, M Zhu, K Heyll, J Corbaln-Campos, P Hartmann, A Thiemann, C Weber, and A Schober. The microRNA-342-5p

fosters inflammatory macrophage activation through an akt1- and microRNA-155-dependent pathway during atherosclerosis. *Circulation*, 127(15):1609–1619, 2013.

[51] Y Wei, A Schober, and C Weber. Pathogenic arterial remodeling: the good and bad of microRNAs. *Am J Physiol Heart Circ Physiol*, 304(8):H1050–H1059, 2013.

[52] Q Wang, Y C Li, J Wang, J Kong, Y Qi, R J Quigg, and X Li. mir-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2 p130. *Proc Natl Acad Sci USA*, 105(8):2889–2894, 2008.

[53] T Sun, M Fu, A L Bookout, S A Kliewer, and D J Mangelsdorf. MicroRNA let-7 regulates 3T3-L1 adipogenesis. *Mol Endocrinol*, 23(6):925–931, 2009.

[54] Y J Kim, S J Hwang, Y C Bae, and J S Jung. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. *Stem Cells*, 27(12):3093–3102, 2009.

[55] C Esau, X Kang, E Peralta, E Hanson, E G Marcusson, L V Ravichandran, Y Sun, S Koo, R J Perera, R Jain, N M Dean, S M Freier, C F Bennett, B Lollo, and R Griffey. MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem*, 279(50):52361–52365, 2004.

[56] S Bork, P Horn, M Castoldi, I Hellwig, A D Ho, and W Wagner. Adipogenic differentiation of human mesenchymal stromal cells is down-regulated by microRNA-369-5p and up-regulated by microRNA-371. *J Cell Physiol*, 226(9):2226–2234, 2011.

[57] I Gerin, G T Bommer, C S McCoin, K M Sousa, V Krishnan, and O A MacDougald. Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis. *Am J Physiol Endocrinol Metab*, 299(2):E198–206, 2010.

[58] E K Lee, M J Lee, K Abdelmohsen, W Kim, M M Kim, S Srikantan, J L Martindale, E R Hutchison, H H Kim, B S Marasa, R Selimyan, J M Egan, S R Smith, S K Fried, and M Gorospe. miR-130 suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor gamma expression. *Mol Cell Biol*, 31(4):626–638, 2011.

[59] Q Lin, Z Gao, R M Alarcon, J Ye, and Z Yun. A role of miR-27 in the regulation of adipogenesis. *FEBS J*, 276(8):2348–2358, 2009.

[60] Y Liu, Q Chen, Y Song, L Lai, J Wang, H Yu, X Cao, and Q Wang. MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation. *FEBS Lett*, 585(12):1963–1968, 2011.

[61] M Karbiener, C Fischer, S Nowitsch, P Opriessnig, C Papak, G Ailhaud, C Dani, E Z Amri, and M Scheideler. microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. *Biochem Biophys Res Commun*, 390(2):247–251, 2009.

[62] S Y Kim, A Y Kim, H W Lee, Y H Son, G Y Lee, J W Lee, Y S Lee, and J B Kim. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. *Biochem Biophys Res Commun*, 392(3):323–328, 2010.

[63] A Ventura, A G Young, M M Winslow, L Lintault, A Meissner, S J Erkeland, J Newman, R T Bronson, D Crowley, J R Stone, R Jaenisch, P A Sharp, and T Jacks. Targeted deletion reveals essential and overlapping functions of the miR-17-92 family of miRNA clusters. *Cell*, 132(5):875–886, 2008.

[64] C Xiao, L Srinivasan, D P Calado, H C Patterson, B Zhang, J Wang, J M Henderson, J L Kutok, and K Rajewsky. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol*, 9(4):405–414, 2008.

[65] L Fontana, E Pelosi, P Greco, S Racanicchi, U Testa, F Liuzzi, C M Croce, E Brunetti, F Grignani, and C Peschle. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol*, 9(7):775–787, 2007.

[66] G Ghosh, I V Subramanian, N Adhikari, X Zhang, H P Joshi, D Basi, Y S Chandrashekhar, J L Hall, S Roy, Y Zeng, and S Ramakrishnan. Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-alpha isoforms and promotes angiogenesis. *J Clin Invest*, 120(11):4141–4154, 2010.

[67] A Rosa, M Ballarino, A Sorrentino, O Sthandier, F G De Angelis, M Marchioni, B Masella, A Guarini, A Fatica, C Peschle, and I Bozzoni. The interplay between the master transcription factor PU-1 and miR-424 regulates human monocyte macrophage differentiation. *Proc Natl Acad Sci U S A*, 104(50):19849–19854, 2007.

[68] K D Taganov, M P Boldin, K J Chang, and D Baltimore. Nf-kappaB-dependent induction of microRNA mir-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*, 103(33):12481–12486, 2006.

[69] R M O'Connell, D S Rao, A A Chaudhuri, M P Boldin, K D Taganov, J Nicoll, R L Paquette, and D Baltimore. Sustained expression of microRNA-155 in

hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*, 205(3):585–594, 2008.

[70] E Tili, J J Michaille, A Cimino, S Costinean, C D Dumitru, B Adair, M Fabbri, H Alder, C G Liu, G A Calin, and C M Croce. Modulation of mir-155 and mir-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol*, 179(8):5082–5089, 2007.

[71] F Bazzoni, M Rossato, M Fabbri, D Gaudiosi, M Mirolo, L Mori, N Tamassia, A Mantovani, M A Cassatella, and M Locati. Induction and regulatory function of mir-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A*, 106(13):5282–5288, 2009.

[72] F J Sheedy, E Palsson-McDermott, E J Hennessy, C Martin, J J O'Leary, Q Ruan, D S Johnson, Y Chen, and L A O'Neill. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA mir-21. *Nat Immunol*, 11(2):141–147, 2010.

[73] H Bao, H Chen, X Zhu, M Zhang, G Yao, Y Yu, W Qin, C Zeng, K Zen, and Z Liu. MiR-223 down-regulation promotes glomerular endothelial cell activation by up-regulating importin a4 and a5 in IGA nephropathy. *Kidney Int*, 85:624–635, 2014.

[74] Q Lili. Association between polymorphisms in RAPGEF1, TP53, NRF1 and type 2 diabetes in chinese han population. *Diabetes Res Clin Pract*, 91:171–176, 2011.

[75] D B Savage. PPARγ as a metabolic regulator: insights from genomics and pharmacology. *Expert Rev Mol Med*, 7:1, 2005.

[76] S Badve. Expression of invariant chain (CD 74) and major histocompatibility complex (MHC) class II antigens in the human fetus. *J Histochem Cytochem*, 50(4):473–482, 2002.

[77] D Starlets. Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. *Blood*, 107:4807–4816, 2006.

[78] A Neerincx. NLRC5, at the heart of antigen presentation. *Front Immunol*, 4:397, 2013.

[79] K Leemasawatdigul and H Gappa-Fahlenkamp. Development of a mathematical model to describe the transport of monocyte chemoattractant protein-1 through a three dimensional collagen matrix. *Cardiovasc Pathol*, 21(3):219–228, 2012.

[80] M Perrot-Applanat. Similar NF-κB gene signatures in TNF-a treated human endothelial cells and breast tumor biopsies. *PlosOne*, 6(7), 2011.

[81] S Herbst, U E Schaible, and B E Schneider. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *Plos One*, 6(5), 2011.

[82] S J Noppert, K A Fitzgerald, and P J Hertzog. The role of type I interferons in TLR responses. *Immunol Cell Biol*, 85:446–457, 2007.

[83] Y V N Cavalcanti. Role of TNF-alpha, IFN-gamma, and IL-10 in the development of pulmonary tuberculosis. *Pulm Med*, 2012.

[84] M J Walter. Interleukin 12 p40 production by barrier epithelial cells during airway inflammation. *J. Exp. Med.*, 193(3), 2001.

[85] H Xu. The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology*, 125:218–228, 2008.